

## Erythropoietin Forms with Improved Properties

IASAT

### Field of the invention

The present invention relates to novel erythropoietin forms such as fusion proteins comprising a Fc portion of an Ig molecule and a molecule having the biological activity of erythropoietin (EPO). By selective altering of the amino acid sequences of the erythropoietin moiety as well as of the immunoglobulin moiety and the glycosylation pattern of erythropoietin, fusion proteins (Fc-EPO) and non-fused EPO with improved properties, e.g. enhanced biological activity and stability, can be obtained. Furthermore, fusion proteins can be provided, wherein shortened versions of erythropoietin and the immunoglobulin chain are used. The invention relates also to novel (non-fused) EPO molecules which have a pattern of cysteines and disulfide bonding which is distinct from human or animal EPO.

### Background

Erythropoiesis, the production of red blood cells, occurs continuously throughout the human life span as a compensation for cell destruction. Erythropoiesis is a precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available in the blood for proper tissue oxygenation, but not so many that the cells would impede circulation. The maturation of red blood cells is under the control of the hormone, erythropoietin (EPO).

Erythropoietin is a acidic glycoprotein hormone of approximately 34,000 daltons. Naturally occurring erythropoietin is produced by the liver during fetal life and by the kidney in response to hypoxia (e.g., red blood cell loss due to anemia) and regulates red blood cell growth and differentiation through interaction with its cognate cellular receptor cells into erythrocytes. It is essential for regulating levels of red blood cells in blood circulation of adults and stimulates the production of red blood cells in bone marrow. Anemia is a consequence of renal failure to produce erythropoietin. Recombinant erythropoietin produced by genetic engineering techniques involving the expression of a protein product from a host cell transformed with the gene encoding erythropoietin has been found to be effective when used in the treatment of anemia resulting from chronic renal failure. Wild type, or naturally-occurring, erythropoietin is defined herein to include

recombinant erythropoietin (Jacobs, K., *et al.*, *Nature*, 313:806-813 (1985)), or naturally-occurring erythropoietin which has been isolated and purified from blood (Miyake, T., *et al.*, *J. Biol. Chem.*, 252:5558-5564 (1977)) or sheep plasma (Goldwasser, E., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 68:697-698 (1971)), or

5 chemically synthesized erythropoietin which can be produced using techniques well known to those of skill in the art. Human erythropoietin is a 166 amino acid polypeptide that exists naturally as a monomer (Lin, F-K., *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7580-7584 (1985)). The tertiary structure of erythropoietin as an isolated protein and in a complex with its receptor has been reported (Syed RS,

10 *et al.*, *Nature* [1998] 395:511-6; Cheetham JC, *Nat Struct Biol.* [1998] 5:861-6). The identification, cloning, and expression of genes encoding erythropoietin are described in U.S. patent 4,703,008. A description of the purification of recombinant erythropoietin from cell medium that supported the growth of mammalian cells containing recombinant erythropoietin plasmids for example, is

15 included in U.S. patent 4,667,016. The expression and recovery of biologically active recombinant erythropoietin from a mammalian cell containing the erythropoietin gene on a recombinant plasmid has, made available quantities of erythropoietin suitable for therapeutic applications. In addition, knowledge of the gene sequence and the availability of larger quantities of purified protein has led

20 to a better understanding of the mode of action of this protein. Several forms of anemia, including those associated with renal failure, HIV infection, blood loss and chronic disease can be treated with this hematopoietic growth factor. Erythropoietin is typically administered by intravenous or subcutaneous injection three times weekly at a dose of approximately 25-100

25 U/kg.

Unlike proteins from prokaryotic cells, many cell surface and secretory proteins produced by eukaryotic cells are modified with one or more oligosaccharide groups. This modification, referred to as glycosylation, can dramatically affect the

30 physical properties of proteins and can also be important in protein stability, pharmacokinetics, secretion, and subcellular localization. Proper glycosylation can be essential for biological activity. In fact, some genes from eukaryotic organisms, when expressed in bacteria (e.g., *E. coli*) which lack cellular processes for glycosylating proteins, yield proteins that are recovered with little or

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One problem with erythropoietin therapy is that, although quite effective, this form of therapy is very expensive. Another problem encountered in the practice of medicine when using injectable pharmaceuticals is the frequency at which those injections must be made in order to maintain a therapeutic level of the compound in the circulation. For example, erythropoietin has a relatively short plasma

half-life (Spivak, J.L., and Hogans, B.B., *Blood*, 73:90 (1989); McMahon, F.G., et al., *Blood*, 76:1718(1990)), therefore, therapeutic plasma levels are rapidly lost, and repeated intravenous administrations must be made.

- 5 It would be advantageous to have available derivatives of erythropoietin which have an extended circulating half-life to avoid such problems. In addition one would prefer to synthesize EPO in a host cell other than a mammalian cell. Unfortunately, synthesis in bacteria is problematic because the protein is not produced in a properly folded, native conformation. Synthesis in insect cells or
- 10 plant cells is also problematic because these cells provide an unfavorable glycosylation pattern. Proteins that are glycosylated according to the insect pattern or the plant patterns are, upon injection into animals, generally taken up by specific receptors and rapidly degraded. For example, macrophages in the liver possess high mannose receptors and asialo-glycoprotein receptors that
- 15 remove proteins with non-mammalian glycosylation patterns.

### Summary of the invention

- The invention provides novel modified EPO forms, above all fusion proteins but
- 20 also non-fused EPO modifications, with surprising activities that address the above-said problems.

- Fusion proteins and modification of specified fusion proteins are known in the art. For example, fusion proteins may effectively block a proteolytic enzyme from
- 25 physical contact with the protein backbone itself, and thus prevent degradation. Additional advantages include, under certain circumstances, improved yield in a specific expression system, correct folding of a target protein, and increasing the stability, circulation time, and the biological activity of the therapeutic protein. One such modification is the use of the Fc region of immunoglobulins. Antibodies
- 30 comprise two functionally independent parts, a variable domain known as "Fab", which binds antigen, and a constant domain, known as "Fc" which provides the link to effector functions such as complement or phagocytic cells.
- The Fc portion of an immunoglobulin mediates a long plasma half life when fused to certain proteins that have particularly short half lives, whereas the mere Fab

fragment is short-lived. Capon, et al., Nature 337: 525-531 (1989). For example, IL-10, an anti-inflammatory and anti-rejection agent has been fused to the N-terminus of murine Fc $\gamma$ 2a in order to increase the cytokine's short circulating half-life (Zheng, X. et al., The Journal of Immunology, 154: 559C-5600 (1995)).

- 5 In addition, the N-terminus of interleukin 2 has also been fused to the Fc portion of IgG1 or IgG3 to overcome the short half life of interleukin 2 and its systemic toxicity (Harvill et al., Immunotechnology, 1: 95-105 (1995)). IL-10 and IL-2, unlike EPO, are small proteins that have very short serum half-lives because they are rapidly cleared by renal filtration.

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Therapeutic fusion proteins have also been constructed using the Fc domain to to incorporate functions such as Fc receptor binding, protein A binding, complement fixation and placental transfer which all reside in the Fc proteins of

- 15 to the N-terminal end of CD30-L, a molecule which binds CD30 receptors expressed on Hodgkin's Disease tumor cells, anaplastic lymphoma cells, T-cell leukemia cells and other malignant cell types (U.S. Patent No. 5,480,981).

- Furthermore, it has been reported in 1996 that efficient expression and secretion of certain non-mutant target proteins can be achieved by expression of fusion  
20 proteins comprising an Fc portion of an immunoglobulin and said target proteins followed by proteolytic cleavage of the target protein (WO 96/08570, US 5,541,087).

- The invention presents novel proteins that have erythropoietin-like activity in their  
25 ability to stimulate production of red blood cells in an animal, but with additional advantageous properties such as increased activity, the ability to be synthesized without glycosylation and longer serum half-life. These novel proteins include mutated versions of EPO which are not fused to other proteins, fusion proteins of EPO to immunoglobulin regions, forms of EPO with altered glycosylation, forms  
30 of EPO that usefully combine mutation, fusion to other moieties, and/or altered glycosylation, forms of EPO that have a truncated amino acid sequence, forms of Fc immunoglobulin portions which are modified / mutated having herewith a reduced affinity e.g. to Fc receptors, shortened or truncated forms of Fc and Fc-EPO constructs having specific linkers.

The EPO forms as defined above and below such as Fc-EPO fusion proteins of this invention show improved properties such as enhanced biological activity and improved stability.

## 5 Detailed Description

It is an object of the present invention to provide a modified erythropoietin (EPO) form having improved properties, wherein said EPO form can be either a non-fused human or mammalian modified EPO having the pattern of cysteines or disulfide bonds that differs from the disulfide bonding or cysteine pattern of human or mammalian EPO, or a fusion protein comprising a Fc portion of an Ig molecule and an erythropoietin molecule (EPO), wherein said Fc portion is fused covalently via its C-terminus directly or indirectly to said EPO molecule by its N-terminus and wherein the Fc portion as well as the EPO portion may be modified or mutated, selected from the group:

- (i) Fc - EPO
- (ii) Fc - L - EPO
- (iii) Fc - EPO<sub>desial</sub>
- (iv) Fc - EPO<sub>m</sub>
- 20 (v) Fc<sub>m</sub> - EPO
- (vi) Fc<sub>m</sub> - EPO<sub>m</sub>
- (vii) Fc<sub>m</sub> - L - EPO
- (viii) Fc - L - EPO<sub>m</sub>
- (ix) Fc - EPO<sub>trunc</sub>
- 25 (x) Fc - L - EPO<sub>trunc</sub>

Herein, EPO has the meaning of naturally occurring EPO from mammalian, preferably human origin and includes also recombinant EPO engineered from natural sources. This EPO according to the invention is glycosylated, non-glycosylated, partially glycosylated or otherwise modified in its glycosylation pattern as indicated above, below and in the prior art. For certain uses, the EPO moiety has a correctly folded structure. The invention discloses novel methods for synthesizing forms of EPO that are unglycosylated. Previously, it was known that glycosylated EPO could be treated with N-glycosidase, which removes sugar

5 are obtained as denatured proteins in inclusion bodies, and do not have disulfide bonds. Thus, additional effort is required to reconstitute the protein into a soluble state. Finally, mutation of the glycosylation sites in EPO results in a protein that cannot be synthesized in mammalian cells (Dube et al., JBC [1988] 263:17516). It appears that the mutant protein is degraded before it can be secreted.

10 However, as disclosed herein, when DNA constructs encoding Fc-unglycosylated EPO are placed in mammalian cell lines, the Fc-unglycosylated EPO is efficiently expressed, secreted, and found in a soluble form in the culture supernatant. The Fc-unglycosylated EPO fusion protein can be purified by standard techniques, for example, on a protein A column. For example, the Fc-unglycosylated EPO can be  
15 injected into animals as an antigen to raise antibodies that are directed against the novel epitopes revealed by the absence of glycosylation. In addition, the Fc-unglycosylated EPO, containing only mutations at the glycosylation sites, has detectable EPO activity and can be used as a starting point for the isolation of additionally mutated forms that have increased activity.

EPO<sub>desial</sub> is a glycosylated EPO according to the invention, wherein sialic acid residues that are normally found on a secreted, glycosylated protein are partially or substantially absent. This can be achieved by enzymatically treatment with an enzyme such that the sialic acid residues have been substantially removed. For example, a protein that is treated with the enzyme neuraminidase will have its sialic acids removed. Such a protein is also recognized by the asialoglycoprotein receptor in the liver. A desialylation can also be achieved by using mutated cells which are deficient in enzymes responsible for this step. For example, the known Lec-2 mutant derivative of the CHO cell line is defective in addition of sialic acid residues to N-linked and O-linked sugar chains in secreted proteins ("asialo"). As a result, the exposed galactose residue on such proteins can be recognized by the asialoglycoprotein receptor in the liver, taken up into cells, and is usually degraded. The desialylation in the EPO moiety of the fusion proteins according to the invention does not need to be completely removed.

EPO<sub>trunc</sub> is an EPO according to this invention which is truncated but not mutated in its amino acid sequence. Truncated forms are protein fragments having essentially the full or only a slightly reduced biological activity of erythropoietin.

Preferred truncated forms of EPO according to this invention are shortened at the

5 C-terminus and have at least 65 amino acids calculated from the N-terminal.

Preferred truncated EPO forms have 155 – 116, 108, 104, 98, 93, 88, 85 or 78 amino acids. Especially preferred forms of EPO ends C-terminally with amino acid positions 108, 104, 98, 93, 88, 85 or 78.

- 10 The Fc region of an immunoglobulin is the amino acid sequence for the carboxyl-terminal portion of an immunoglobulin heavy chain constant region. The Fc regions are particularly important in determining the biological functions of the immunoglobulin and these biological functions are termed effector functions. As known, the heavy chains of the immunoglobulin subclasses comprise four or five
- 15 domains: IgM and IgE have -five heavy chain domains, and IgA, IgD and IgG have four heavy chain domains. The Fc region of IgA, IgD and IgG is a dimer of the hinge-CH<sub>2</sub>-CH<sub>3</sub> domains, and in IgM and IgE it is a dimer of the hinge-CH<sub>2</sub>-CH<sub>3</sub>-CH<sub>4</sub> domains (see, W.E.Paul, ed., 1993, Fundamental Immunology, Raven Press, New York, New York).

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As used herein, the term "Fc portion of an Ig molecule" means the carboxyl-terminal portion of an immunoglobulin heavy chain constant region, or an analog or portion thereof. That is, e.g., an immunoglobulin Fc region of Ig, preferably IgG, most preferably IgG1, IgG2 and IgG3, may comprise at least a portion of a

25 hinge region, a CH<sub>2</sub> domain, and a CH<sub>3</sub> domain. In a preferred embodiment the Fc region includes at least a portion of a hinge region and a CH<sub>3</sub> domain.

In some circumstances, it is useful to mutate certain amino acids within the Fc moiety of an Fc-EPO fusion protein. For example, if an Fc-EPO fusion protein is

30 to be expressed in a cell type that generates a non-human glycosylation pattern, it is often useful to mutate the glycosylation site within the Fc region and thus entirely eliminate glycosylation at this site. As a result, the resulting protein will not be identified and degraded by scavenging systems that recognize altered glycosylation patterns.



Thus, Fc<sub>m</sub> is a Fc portion as defined above which is mutated and / or truncated in its amino acid sequence and / or modified in its glycosylation pattern. It has been shown by this invention that such modified Fc portions lead to Fc-EPO fusion proteins with improved properties. In this context Fc<sub>m</sub> includes additionally

5 modified or mutated Fc portions which have a reduced affinity to Fc receptors on cells. The binding affinity of fusion proteins for Fc receptors can be reduced by using heavy chain isotypes as fusion partners that have per se diminished binding affinity for Fc receptors on cells. For example, it is known that, for example, IgG1 and IgG3 bind to FcRγ1 with high affinity and that the binding sites

10 are located in the CH2 domain. Thus, it is an object of the invention to provide a Fc-EPO fusion protein with enhanced in vivo circulating half-life having a mutation, deletion or insertion at one or more amino acids in the domains responsible for Fc receptor binding. In a preferred embodiment of the invention the Fc-EPO fusion protein comprises a Fc portion of an IgG1, wherein said

15 mutations, deletions, or insertions in the IgG1 constant region are selected from Leu<sub>234</sub>, Leu<sub>235</sub>, Gly<sub>236</sub>, Gly<sub>237</sub>, Asn<sub>297</sub>, and Pro<sub>331</sub>. In an alternative preferred embodiment the mutation, deletion or insertion is introduced in the IgG1 constant region of a Fc portion of a fusion protein according to the invention at one or more amino acids selected from Leu<sub>281</sub>, Leu<sub>282</sub>, Gly<sub>283</sub>, Gly<sub>284</sub>, Asn<sub>344</sub> and Pro<sub>378</sub>.

20 Methods for making Fc portions with reduced Fc receptor affinity are, for example, disclosed in PCT/US99/03966.

The invention also discloses methods for generating useful mutant forms of Fc-EPO in which the EPO moiety is altered. Variants of Fc-EPO with increased EPO

25 biological activity can be generated by procedures described in the Examples and known in the art.

Thus, EPO<sub>m</sub> is an EPO according to this invention which is mutated but not truncated in its amino acid sequence. The number of mutations is not limited but is restricted to the loss of the biological activity of the molecule. Preferably

30 mutations of 1 to 10 amino acids are used. Surprisingly it could be shown that the Fc fusion proteins according to the invention, wherein EPO is mutated as defined above, have greater specific activity than the comparable Fc-EPO fusion proteins having no mutated EPO moieties. Therefore, it is an preferred object of the invention to provide Fusion proteins as defined above and in the claims, wherein

EPO is mutated. Preferred fusion proteins of this invention have an EPO molecule, wherein in the EPO<sub>m</sub> portion at least one of the following changes are achieved: Asn<sub>24, 38, 83</sub> → Xxx, Ser<sub>126</sub> → Xxx, where Xxx is a different amino acid. Preferred changes according to the invention are Asn<sub>24, 38, 83</sub> → Gln and / or  
5 Ser<sub>126</sub> → Ala. Further preferred mutations are: His 32 → Gly and / or Ser 34 → Arg and / or Pro 90 → Ala. In one embodiment of the invention all above-said mutations are achieved.

These and other variant proteins according to the invention may enhance binding  
10 to the EPO receptor, enhanced stability, enhanced adoption of a correct, active conformation, enhanced pharmacokinetic properties, enhanced synthesis, or other advantageous features. Mutations in the EPO moiety of Fc-EPO can be combined to generate proteins that have further enhanced activities.  
A specific method for improvement of Fc-EPO disclosed in the Examples uses  
15 site-directed mutagenesis techniques. It is important to note that a wide variety of site-directed mutagenesis techniques are available, and can be used as alternatives to achieve similar results. The strategies for choosing among these techniques is well-known to those skilled in the art of molecular biology.  
Similarly, there is a wide variety of techniques for achieving random and semi-  
20 random mutagenesis of a target DNA. These techniques are also well-known to those skilled in the art of molecular biology.

Additional mutant forms of Fc-EPO may be constructed according to this invention. The mutations have the effect of increasing the activity of  
25 unglycosylated Fc-EPO. Depending on the mutation, activity is increased by a variety of mechanisms, such as increasing affinity of Fc-EPO for the EPO receptor, increasing the fraction of Fc-EPO that is properly folded, or improving the pharmacokinetic properties of Fc-EPO. Some mutations, when combined, have an additive or multiplicative effect on the activity of unglycosylated Fc-EPO.

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The Fc portion and the EPO proteins according to this invention may also be linked by linker molecules, wherein the chemical or amino acid linkers are of varying length. The chemical linkers are well known in the art. Peptide linkers are

preferred. Fusion proteins, wherein the Fc portion is linked with the target protein by a linker molecule may have improved properties. The Fc- EPO fusion proteins according to the invention having such linker molecules show an enhanced biological activity. The linker of the invention (L) is a linker molecule as defined  
 5 above and below which has no protease cleavage site.

The peptide linker often is a series of peptides such as. e.g., glycine and/or serine. Preferably, the peptide linker is a mixed series of glycine and serine peptides about 5 - 25, preferably 10 - 20 residues in length.

~~Preferred amino acid linkers L are used and include the following sequences,~~  
 10 wherein such linkers are excluded that possess a cleavage site for proteolytic enzymes:

1. Ala Ala Ala
2. Ala Ala Ala Ala,
3. Ala Ala Ala Ala Ala,
- 15 4. Ser,
5. Ser Ser,
6. Gly Gly Gly,
7. Gly Gly Gly Gly,
8. Gly Gly Gly Gly Gly,
- 20 9. Gly Gly Gly Gly Gly Gly Gly,
10. Gly Pro Gly,
11. Gly Gly Pro Gly Gly,
12. Gly Gly Gly Gly Ser, and
13. any combinations of subparts 1-12
- 25 Preferred amino acid linkers are (Gly Gly Gly Gly Ser)<sub>x</sub> wherein x is 1-5.

~~Additional suitable linkers are disclosed in Robinson et al., 1998, Proc. Natl. Acad. Sci. USA, 95, 5929.~~

As used herein, "proteolytic cleavage site" means amino acid sequences which  
 30 are preferentially cleaved by a proteolytic enzyme or other proteolytic cleavage agents. Proteolytic cleavage sites include amino acids sequences which are recognized by proteolytic enzymes such as trypsin, plasmin or enterokinase K. Many cleavage site/cleavage agent pairs are known. Where the target protein sequence is a precursor molecule to Interferon-alpha or an active variant thereof,

As used above and below, the biological activity of erythropoietin and EPO fusion proteins, respectively, is defined as the ability to regulate red blood cell growth

To be "functionally" or "biologically active" an erythropoietin-like molecule, such as Fc-EPO, typically shares substantial sequence (amino acid) similarity (e.g., at least about 65%, typically at least about 80% and most typically about 90 - 95%) with the corresponding sequences of wild type, or naturally-occurring, erythropoietin and possesses one or more of the functions of wild type erythropoietin thereof.

Another and important aspect of this invention is the finding that in order to get improved EPO forms, preferably Fc-EPO fusion proteins, it is advantageous to introduce altered patterns of cysteine-cysteine disulfide bonds. Thus, it is an object of the invention to provide Fc-EPO fusion proteins or non-fused EPO, wherein at least one, preferably 2 – 4 cystein residues of the EPO or EPO<sub>m</sub> moiety are engineered. Especially, it is an object of the invention to provide Fc-EPO fusion proteins or non-fused EPO, wherein the EPO or EPO<sub>m</sub> moiety has a pattern of disulfide bonding which is distinct from human or mammalian EPO. In one embodiment of the invention the EPO moiety includes one or more of the following amino acid variations: position 29 is not Cys, position 33 is not Cys, position 88 is Cys and position 139 is Cys. In a preferred Cys - engineered

embodiment of this invention the EPO moiety is derived from human EPO and has at least one of the following mutations: His 32 → Gly, Ser 34 → to Arg and Pro 90 → Ala.

To generate an altered disulfide bond, one cysteine residue is mutated to a structurally compatible amino acid such as alanine or serine, and a second amino acid that is nearby in the three-dimensional structure is mutated to cysteine. Thus, it is a further object of this invention to provide FC-EPO fusion proteins or non-fused EPO, wherein at least one of the cysteine residues of the EPO molecule or EPO<sub>m</sub> molecule is engineered by techniques which are well known in the art. One embodiment is a Fc-EPO fusion protein, wherein Cys<sub>33</sub> is replaced by any other amino acid. In an alternative embodiment a fusion protein is object of the invention, wherein one of the amino acids Gln<sub>86</sub>, Pro<sub>87</sub>, Trp<sub>88</sub>, Glu<sub>89</sub>, Leu<sub>91</sub> is replaced by Cys. Preferably, Trp<sub>88</sub> is replaced by Cys. For example, a fusion protein containing an EPO moiety lacking Cys at position 33 and containing Cys at position 88 will form a disulfide bond that is not found in human EPO. This bond results in a fusion protein that has superior properties to an otherwise similar fusion protein containing a disulfide bond between Cys<sub>29</sub> and Cys<sub>33</sub>. For example, the Cys<sub>29</sub>-Cys<sub>88</sub> fusion protein has greater activity than the Cys<sub>29</sub>-Cys<sub>33</sub> fusion protein. In addition, the Cys<sub>29</sub>-Cys<sub>88</sub> fusion protein shows a pronounced increase in activity, relative to the Cys<sub>29</sub>-Cys<sub>33</sub> fusion protein, in the presence of other mutations in the EPO moiety of the fusion protein. It is also sometimes useful to incorporate the mutations His<sub>32</sub> to any other amino acid, preferably Gly or Ser, mutation of Ser<sub>34</sub> to Arg, and mutation of Pro<sub>90</sub> to Ala.

Another useful set of mutations includes mutation of Cys<sub>29</sub> of the EPO of the invention to any other amino acid, and mutation of Arg<sub>139</sub> to Cys. An EPO form containing both of these mutations will generally contain a disulfide bond between Cys<sub>33</sub> and Cys<sub>139</sub>. This bond results in a fusion protein that has superior properties to an otherwise similar fusion protein containing a disulfide bond between Cys<sub>29</sub> and Cys<sub>33</sub>. For example, the Cys<sub>33</sub>-Cys<sub>139</sub> fusion protein has greater activity than the Cys<sub>29</sub>-Cys<sub>33</sub> fusion protein. In addition, the Cys<sub>33</sub>-Cys<sub>139</sub> fusion protein shows a pronounced increase in activity, relative to the Cys<sub>29</sub>-Cys<sub>33</sub>

fusion protein, in the presence of other mutations in the EPO moiety of the fusion protein.

As a further alternative, an entirely new disulfide bond is added to the protein by mutating two amino acids to cysteines.

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It is known in the art that erythropoietin synthesized by non-human animals generally contains a different pattern of cysteine residues than human erythropoietin (Wen, D., et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals Blood 82, 1507-1516 [1993]; Fu, P., et al. The sheep erythropoietin gene: molecular cloning and effect of hemorrhage on plasma erythropoietin and renal/liver messenger RNA in adult sheep Mol. Cell. Endocrinol. 93, 107-116 [1993]; Lin, F.K., et al., Monkey erythropoietin gene: cloning, expression and comparison with the human erythropoietin gene Gene 44, 201-209 [1986]; Suliman, H.B., et al. Cloning of a cDNA encoding bovine erythropoietin and analysis of its transcription in selected tissues Gene 171, 275-280 (1996) ; McDonald, J.D., Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene Mol. Cell. Biol. 6, 842-848 [1986]; Nagao, M., et al. Nucleotide sequence of rat erythropoietin Biochim. Biophys. Acta 1171 (1), 99-102 [1992]). However, the erythropoietin normally produced by most of these animals, such as macaques, pigs, dogs, cats, cows, and sheep, contains five cysteines. Rodents such as mice and rats have four cysteines, but the two of the cysteines are at positions 29 and 139. Based on the three-dimensional structure of human EPO, the cysteines of rodent EPO at positions 29 and 139 are unable to form disulfide bonds with each other or any of the other cysteines. In general, secreted extracellular proteins do not contain unpaired cysteines. In the relatively oxidizing environment of the extracellular space, unpaired cysteines may be oxidized, for example, to cysteic acid. As a result, the activity of the protein may be reduced. Without wishing to be bound by theory, the oxidation of cysteines in the EPO of non-human animals may serve to down-regulate EPO activity, inactivating the EPO protein in conditions of high oxygen when erythropoiesis is not needed.

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In any case, the invention provides EPO moieties that are distinct from known animal-derived forms of EPO in that they have an even number of cysteines and

all of the cysteines are capable of forming disulfide bonds. These EPO moieties containing novel disulfide bonding patterns may be useful as Fc fusions, as fusions to other proteins such as albumin, or as unfused, isolated moieties.

- 5 Another feature of the invention is a form of EPO with cysteines at positions 29, 33, 88, and 139. When this set of cysteines is present in an EPO that contains the usual cysteines at positions 7 and 161, the resulting EPO contains three disulfide bonds instead of two. The resulting molecule is extremely stable, even in the presence of other mutations that destabilize the normal EPO protein. For
- 10 example, EPO (Cys<sub>29</sub>-Cys<sub>88</sub>, Cys<sub>33</sub>-Cys<sub>139</sub>) is much more stable than normal human EPO. Similarly, fusion proteins such as Fc fusions to EPO (Cys<sub>29</sub>-Cys<sub>88</sub>, Cys<sub>33</sub>-Cys<sub>139</sub>) are more stable than the corresponding fusions to normal human EPO or to normal EPO from non-human animals.
- 15 Thus, the invention presents novel forms of EPO and EPO fused to other moieties, preferably an Fc moiety, that have patterns of cysteine residues and disulfide bonds that are different from human and animal EPO. These novel forms of EPO have significant advantages over corresponding natural forms of EPO. For example, forms of EPO with altered disulfide bonding patterns have
- 20 higher specific activity, increased stability, dramatically increased stability in the presence of other alterations that destabilize EPO, and improved pharmacokinetics. Some of the Examples below illustrate these points. For example, enzymatic deglycosylation of EPO has a destabilizing effect on EPO activity. A form of EPO with an altered pattern of disulfide bonds is more stable
- 25 upon deglycosylation than the corresponding form of EPO with the normal disulfide bonding pattern. In addition, a form of EPO with an altered pattern of disulfide bonding has a greater specific activity than the corresponding form of EPO with a normal pattern of disulfides.
- 30 Thus, it is also an object of the present invention to provide a novel recombinant human or animal preferably mammalian (non-Fc-fused) erythropoietin (EPO) having the pattern of disulfide bonds that differs from the disulfide bonding pattern of human or animal / mammalian EPO. Animal or mammalian EPO according to the invention may derive from mice, macaques, rats, dogs, pigs, cows or sheep.

Furthermore, it is an object of the present invention to provide a fusion protein as defined above and in the claims, wherein the EPO or EPO<sub>m</sub> portions within the Fc fusion protein are dimerized.

The term "dimeric" refers to a specific multimeric molecule, wherein two protein subunits are stably associated through covalent or non-covalent interactions. As used herein, the term "multimeric" refers to the stable association of two or more protein subunits by means of covalent interaction, for example, by a disulfide bond or by means of non-covalent interaction.

It should be understood that the Fc fragment itself typically is a dimer of the heavy chain fragments including at least a portion of the hinge region, CH<sub>2</sub> domain and CH<sub>3</sub> domain. However, many protein ligands are known to bind to their receptors as a dimer. If a protein ligand X dimerizes naturally, the X moiety in a Fc-X molecule will dimerize to a much greater extent, since the dimerization process is concentration dependent. The physical proximity of the two X moieties connected by Fc would make the dimerization an intramolecular process, greatly shifting the equilibrium in favor of the dimer and enhancing its binding to the receptor.

It is another object according to the invention to construct EPO fusion proteins, wherein a whole or intact Ig molecule is used. Such fusion molecules comprise the variable regions of heavy and light chains of an antibody and the epitopes binding to a specific antigen. For example, erythropoietin is fused to the C-terminus of an antibody heavy chain within an antibody whose variable regions are directed against an antigen to which much or all of the human population has been exposed. Such an antibody is termed a "universal antibody" in this disclosure. It is important to note that the use of "universal" antibodies in the construction of antibody fusion proteins can be generalized to fusion molecule with other protein moieties besides erythropoietin. By a "universal" antibody is meant an antibody with a specificity that is found in much, most, or all of a mammalian population, such as the human population.

For example, variable regions directed against tetanus toxoid are encoded in the human genome and the corresponding proteins are generally represented in the serum without having experienced somatic mutation. Thus, according to the invention, erythropoietin is fused to the C-terminus of a heavy chain of an



antibody directed against tetanus toxoid. An advantage of such an antibody-erythropoietin fusion is that the antibody variable regions do not bind strongly to a mammalian self-antigen. A second advantage is that anti-idiotypic antibodies are less likely to be generated de novo against such an antibody than to an antibody  
5 with an uncharacterized variable region.

DNA constructs encoding whole antibody fusion proteins may be constructed as described previously (Gillies et al. [1991] *Hybridoma* 10:347-356).

The invention also relates to a DNA molecule that encodes any of the fusion  
10 proteins disclosed above and depicted in the claims.

As a preferred embodiment a DNA molecule is disclosed that encodes a fusion protein as defined above and in the claims comprising:

- (a) a signal / leader sequence
- 15 (b) a Fc region of an Ig molecule
- (c) a target protein sequence having the biological activity of EPO.

The signal sequence of the invention as indicated above is a polynucleotide which encodes an amino acid sequence that initiates transport of a protein across  
20 the membrane of the endoplasmic reticulum. Signal sequences which will be useful in the invention include antibody light chain signal sequences, e.g., antibody 14.18 [Gillies et. al. (1989) *Jour. of Immunol. Meth.*, 125:19 1 ], antibody heavy chain signal sequences, e.g., the MOPC141 antibody heavy chain signal sequence [Sakano et al. (1980) *Nature* 286:5774], and any other signal  
25 sequences which are known in the art (see for example, Watson, 1984, *Nucleic Acids Research* 12:5145). Each of these references is incorporated herein by reference. Signal sequences have been well characterized in the art and are known typically to contain 16 to 30 amino acid residues, and may contain greater or fewer amino acid residues. A typical signal peptide consists of three regions: a  
30 basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region. The central hydrophobic region contains 4 to 12 hydrophobic residues that anchor the signal peptide across the membrane lipid bilayer during transport of the nascent polypeptide. Following initiation, the signal peptide is

usually cleaved within the lumen of the endoplasmic reticulum by cellular enzymes known as signal peptidases.

Potential cleavage sites of the signal peptide generally follow the "(-3, -1) rule".

Thus a typical signal peptide has small, neutral amino acid residues in positions

- 5 -1 and -3 and lacks proline residues in this region. The signal peptidase will cleave such a signal peptide between the -1 and +1 amino acids. Thus, the portion of the DNA encoding the signal sequence may be cleaved from the amino-terminus of the Fc- fusion protein during secretion. This results in the secretion of a Fc-fusion protein consisting of the Fc region and the target protein.
- 10 A detailed discussion of signal peptide sequences is provided by von Heijne (1986) Nucleic Acids Res., 14:4683. As would be apparent to one of skill in the art, the suitability of a particular signal sequence for use in a secretion cassette may require some routine experimentation. A signal sequence is also referred to as a "signal peptide", "leader sequence" or "leader peptides" and each of these
- 15 terms having meanings synonymous to signal sequence may be used herein.

The invention also relates to expression vectors comprising said DNA molecules which promote expression of the target protein, that is a Fc-EPO fusion protein.

As used herein, "vector" means any nucleic acid comprising a nucleotide

- 20 sequence competent to be incorporated into a host cell and to be recombined with and integrated into the host cell genome, or to replicate autonomously as an episome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors and the like. Non-limiting examples of a viral vector include a retrovirus, an adenovirus and an adeno-associated virus.
- 25 As used herein, "expression of a target protein" is understood to mean the transcription of the DNA sequence, translation of the mRNA transcript, and secretion of a protein product that is folded into a correct, active conformation.

According to the invention eukaryotic, preferably mammalian, host cells are used

- 30 that are suitable for expressing a fusion protein as defined in this application.

Methods of transfecting such host cells with said vector, expressing, purifying and isolating the fusion proteins of this invention are well known in the art.

Therefore, the method according to this invention comprises:

- (i) constructing a DNA encoding a precursor protein that comprises from N-

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terminus to C-terminus a leader sequence for secretion, the Fc portion and the EPO, EPO<sub>m</sub> or EPO<sub>trunc</sub>,

- (ii) placing said fused DNA in an appropriate expression vector,
- (iii) expressing said fusion protein in a eukaryotic cell, and
- 5 (iv) purifying said secreted fusion protein.

Finally, the invention also relates to pharmaceutical compositions comprising at least one of the EPO forms as defined above and below, preferably a Fc-EPO fusion protein, together with pharmaceutically acceptable carriers, diluents, and  
10 excipients. These pharmaceutical compositions may optionally contain other drugs or medicaments that are helpful in co-treating EPO deficient diseases.

Such pharmaceutical compositions may be for parenteral administration, or for oral, pulmonary, nasal, transdermal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising  
15 effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80),  
20 anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol).

The term "parenteral" as mentioned above and below includes subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. The parenteral administration is preferred.

25 As used herein, the term "pharmaceutically acceptable carrier or excipient" means an inert, non toxic liquid filler, diluent, solvent or solution, not reacting adversely with the active compounds or with the patient. Suitable liquid carriers are well known in the art such as steril water, saline, aqueous dextrose, sugar solutions, ethanol, glycols and oils, including those of petroleum, animal,  
30 vegetable, or synthetic origin. The formulations may also contain adjuvants or vehicles which are typical for parenteral administration.

With respect to said suitable formulations it should be pointed out that the Fusion proteins of the present invention may eventually form pharmaceutically

acceptable salts with any non-toxic, organic or inorganic acid showing changed solubility. Inorganic acids are, for example, hydrochloric, sulphuric or phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Examples for organic acids are the mono, di and tri

5 carboxylic acids such as acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, benzoic, phenylacetic, cinnamic, salicylic and sulfonic acids. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. These salts include, for example, alkali metals such

10 as sodium and potassium, alkaline earth metals such as calcium and magnesium, and organic primary, secondary and tertiary amines such as trialkylamines.

Preferably, the dosage of the pharmaceutical composition according to the invention will be such that between about 10 ng / kg / day and about 10 µg / kg /

15 day will yield the desired therapeutic effect. The effective dosages may be determined using diagnostic tools which are known in the prior art. In general, the optimum therapeutically acceptable dosage and dose rate for a given patient within the above-said ranges depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health,

20 sex, diet, time and route of administration, rate of clearance or the object of treatment. One skilled in the art will be able to ascertain effective dosages by administration and observing the desired therapeutic effect. The dosages may also vary over the course of therapy, with a relatively high dosage being used initially, until therapeutic benefit is seen, and lower dosages used to maintain the

25 therapeutic benefits.

### Short Description of the Figures

#### Figure 1:

30 Fc-Erythropoietin treated with N-glycosidase

SDS gel showing Fc-erythropoietin before and after N-glycosidase F treatment. Lane 1 shows molecular weight size standards, lane 2 is blank, lane 3 shows normally glycosylated Fc-EPO, lane 4 shows normally glycosylated Fc-EPO after

incubation in deglycosylation buffer, and lanes 5, 6, and 7 respectively show normally glycosylated Fc-EPO incubated with 20 units of N-glycosidase F (Boehringer-Mannheim) in 0.5 mls for 3 hours, 6 hours, or 18 hours.

5 Figure 2:

This figure depicts a line graph showing the biological activity of the NIBSC EPO (black diamonds), human IgG2 Fc-EPO (white squares), human IgG1 Fc-EPO with mutant glycosylation sites (white circles), and human IgG2 Fc-EPO expressed in CHO-Lec2 cells (white diamonds). The activity of the EPO moiety in various proteins was assayed via EPO-dependent 3H-thymidine incorporation into TF-1 cells. The X-axis indicates the EPO equivalents (ng/ml) present as determined by ELISA, and the Y-axis indicates the dependent 3H-thymidine incorporation in counts per minute.

15 Figure 3:

This figure depicts a line graph showing the biological activity of human IgG2 Fc-EPO treated with neuraminidase for various times. Fc-EPO was treated with buffer alone (black circles), 0.1 units of neuraminidase for 15 minutes (white squares), for 1 hour (white diamonds), for 3.5 hours (white triangles), or for 22 hours (white circles). The activity of the EPO moiety in various proteins was assayed via the EPO-dependent 3H-thymidine incorporation into TF-1 cells. The X-axis indicates the EPO equivalents (ng/ml) present, and the Y-axis indicates the dependent 3H-thymidine incorporation in counts per minute.

25 Figure 4:

Figure 4 depicts an HPLC profile of purified Fc-EPO in which the EPO moiety had the human EPO sequence except for the following alterations: His<sub>32</sub> → Gly, Cys<sub>33</sub> → Pro, Trp<sub>88</sub> → Cys, and Pro<sub>90</sub> → Ala. The peak at 7.064 represents (Fc-EPO)<sub>2</sub>, and the peak at 5.302 represents aggregated material with a molecular weight of at least 800,000 daltons. The peak at 7.064 represents 93.2% of the detected material, while the peak at 5.302 represents 6.8% of the loaded material.

**Figure 5:**

This figure depicts an HPLC profile of purified Fc-EPO in which the EPO moiety had the human EPO sequence. The peak at 7.254 represents (Fc-EPO)<sub>2</sub>, the peak at 6.079 represents an oligomeric aggregate of (Fc-EPO)<sub>2</sub>, and the peak at 5.330 represents aggregated material with a molecular weight of at least 800,000 daltons. The peak at 7.254 represents 43.4% of the detected material, while the peaks at 6.079 and at 5.330 respectively represent 30.5% and 25.2% of the loaded material.

**10 Sequence Information**

The following DNA and amino acid sequences were used in this invention

The coding sequence for mature EPO, using modified codons to optimize translation and including bases at the 5' end comprising the SmaI site is given in

15 Seq. Id. No. 1.

**SEQ ID NO:1**

(Small characters indicate base differences from the human EPO coding sequence that are predicted to increase expression but not change protein sequence. )

20 CCCGGGtGCCCCACCGCCTCATCTGTGACAGCCGAGTgCTGGAGAGGTACCTCT  
TGGAGGCCAAGGAGGCCGAGAATATCACGACcGGCTGTGCTGAACACTGCAGCTT  
GAATGAGAAcATCACcGTgCCtGACACCAAAGTgAATTTCTATGCCTGGAAGAGGATG  
GAGGTtGGcCAGCAGGCCGTAGAAGTgTGGCAGGGCCTGGCCCTGCTGTcGGAAG  
CTGTCCTGCGGGGCCAGGCCCTGTTGGTCAACTCTTCCCAGCCGTGGGAGCCCCT  
25 GCAaCTGCATGTGGATAAAGCCGTgAGTGGCCTTCGCAGCCTCACCACTCTGCTTC  
GGGCTCTGgGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGC  
TCCcCTCCGcACAATCACTGCTGACACTTTCCGCAAACCTCTTCCGAGTCTACTCCAAT  
TTCCTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTgcCGGACAGGGGACA  
GATGActcgag

30

**SEQ ID NO:2**

The mature EPO protein sequence (one-letter code)

APPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNfYAWKRMEVGQQ  
AVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTLLRALGAQKE  
AISPPDAASAAPLRITADTFRKLFRVYSNFLRGKCLKLYTGEACRTGDR

35

Oligonucleotides used to construct a fusion of normally glycosylated EPO to the C-terminus of an Fc region.

SEQ ID 3: Oligo 1

CCGGGtGCCCCACCACGCCTCATCTGTGACAGCCGAGTgCTGGAGAGGTACC

5 SEQ ID 4: Oligo 2

TCTTGAGGCCAAGGAGGCCGAGAATATCACGACcGGCTGTGCTGAACA

SEQ ID 5: Oligo 3

CTGCAGCTTGAATGAGAAcATCACcGTgCCTGACACCAAAGTgAATTTCTAT

SEQ ID 6: Oligo 4

10 GCCTGGAAGAGGATGGAGGTtGGcCAGCAGGCCGTAGAAGTgTGGCAG

SEQ ID 7: Oligo 5

GGCCTGGCCCTGCTGTCTCGGAAGCTGTCCTGCGGGGCCAGGCCCTGTTGGTC

SEQ ID 8: Oligo 6

AACTCTTCCCAGCCGTGGGAGCCCCCTGCAaCTGCATGTGGATAAAGCCG

15 SEQ ID 9: Oligo 7

TgAGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCTGgGAGCCCAGAA

SEQ ID 10: Oligo 8

GGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCcCTCCGcAC

SEQ ID 11: Oligo 9

20 AATCACTGCTGACACTTTCCGCAAACCTCTTCCGAGTCTACTCCAATTTCTCC

SEQ ID 12: Oligo 10

GGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTgcCGGACAGGGGACAGATGActc  
gag

25 Mutagenesis of glycosylation sites:

SEQ ID 13: Oligo 11 (oligo 2')

tc ttggaggcca aggaggccga gcagatcacg acgggctgtg ctgaaca

TCTTGAGGCCAAGGAGGCCGAG**CAG**ATCACGACcGGCTGTGCTGAACA

SEQ ID 14: Oligo 12 (oligo 3')

30 CTGCAGCTTGAATGAG**CAG**ATCACcGTgCCTGACACCAAAGTgAATTTCTAT

SEQ ID 15: Oligo 13 (oligo 6')

**CAG**TCTTCCCAGCCGTGGGAGCCCCCTGCAaCTGCATGTGGATAAAGCCG

SEQ ID 16: Oligo 14 (oligo 8')

GGAAGCCATCTCCCCTCCAGATGCGGCC**CAG**CTGCTCCcCTCCGcAC

SEQ ID 17:

*Human IgG1 Fc region – mature protein coding sequence*

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK  
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI  
5 EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN  
YKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID 18:

*Human IgG2 constant region – mature protein coding sequence (CH1, hinge,  
10 CH2, and CH3 regions)*

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTWWSNGALTSGLV  
HTFPAVLQSSGLYSLSSVVTVPSSNFGTQT YTCNV D HKPS NTKVDKTVR  
KCCVECPPECPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP  
EVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKC  
15 KVSNGKLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG  
FYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGN  
VFSCFSVMHEALHNHYTQKSLSLSPGK

The following examples describe the invention in more detail without limiting it.

20

**Example 1.**Expression of human Fc-EPO

A sequence encoding the mature form of erythropoietin was completely  
synthesized from oligonucleotides by standard techniques. The oligonucleotides  
25 shown above and in the Sequence Listing indicate the 'top' strand of DNA  
encoding EPO protein; 'bottom' strand oligonucleotides were designed to pair  
with the top strands and to generate 4 to 5 base protruding 5' ends so that the  
entire EPO coding sequence could be ligated together after phosphorylation of  
the oligonucleotides. The sequence was designed to have a high G/C content  
30 with optimal codon usage for high-level expression.

The protein according to SEQ ID 2 does not have the N-terminal lysine residue of  
the mature protein. The synthesized DNA was engineered to have an XmaI-  
compatible overhang at the 5' end and an XhoI-compatible overhang at the 3'  
35 end. An alternative sequence was constructed with mutations in the four EPO



glycosylation sites: Asn<sub>24</sub> → Gln, Asn<sub>38</sub> → Gln, Asn<sub>83</sub> → Gln, and Ser<sub>126</sub> → Ala.

The 500 base-pair DNA was cloned and sequence analysis confirmed that it encodes the mature human EPO without additional undesired mutations.

The expression vector pdCs-Fc-EPO was constructed as follows. The XmaI-XhoI  
5 restriction fragment containing the human EPO cDNA was ligated to the XmaI-XhoI fragment of the pdCs-Fc vector according to Lo *et al.* [Protein Engineering (1998) 11:495]. The resultant vector, pdCs-Fc-EPO, was used to transfect mammalian cells for the expression of Fc-EPO. This vector expresses the human immunoglobulin gamma1 chain Fc region. A second set of Fc-EPO vectors were  
10 constructed in which the gamma1 chain Fc region was replaced with an Fc region derived from human gamma2.

The Fc protein moiety also usually contains a glycosylation site. This site may be optionally changed to a non-glycosylated sequence by standard approaches.

## 15 **Example 2.**

### Transfection and expression of Fc-EPO fusion proteins

For transient transfection, the plasmids were introduced into BHK cells. Cells were transfected by coprecipitation of plasmid DNA with calcium phosphate [Sambrook *et al.* (1989) Molecular Cloning--A Laboratory Manual, Cold Spring  
20 Harbor, NY] or by lipofection using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) according to supplier's protocol.

To generate stable cell lines, NS/O cells were used for both transient transfection and the generation of stable cell lines. To express proteins lacking the normal sialic acid modification, CHO-Lec2 cells (ATCC Number: CRL-1736) This cells  
25 exhibit a drastic reduction in the transport of CMP-sialic acid into the Golgi compartment, and are useful for studying the contribution of sialic acid in protein function.

In order to obtain stably transfected clones, plasmid DNA was introduced into cells by electroporation. About  $5 \times 10^6$  cells were washed once with PBS and  
30 resuspended in 0.5 ml PBS. Ten µg of linearized plasmid DNA were then incubated with the cells in a Gene Pulser Cuvette (0.4 cm electrode gap, BioRad) on ice for 10 min. Electroporation was performed using a Gene Pulser (BioRad, Hercules, CA) with settings at 0.25 V and 500 microF. Cells were allowed to

recover for 10 min. on ice, after which they were resuspended in growth medium and then plated onto two 96 well plates. Stably transfected clones were selected by growth in the presence of 100 nM methotrexate (MTX), which was introduced two days post-transfection. The cells were fed every 3 days for two to three more  
5 times, and MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-Fc ELISA to identify high producers. High producing clones were isolated and propagated in growth medium containing 100 nM MTX.

BHK cells and NS/O cells were grown in Dulbecco's modified Eagle's medium  
10 supplemented with 10% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. CHO-Lec2 cells were grown in alpha medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. For routine characterization by gel electrophoresis, Fc fusion proteins in the conditioned media were captured on Protein A Sepharose (Repligen, Cambridge,  
15 MA) and then eluted by boiling in the protein sample buffer with or without 2-mercaptoethanol. After electrophoresis on an SDS gel, the protein bands were visualized by Coomassie staining. Fc-EPO had an apparent MW of about 64 kD on SDS-PAGE. For purification, the fusion proteins bound on Protein A Sepharose were eluted in  
20 a sodium phosphate buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 3, and 150 mM NaCl). The eluate was then immediately neutralized with 0.1 volume of 2 M Tris-hydrochloride, pH 8.

### Example 3.

#### 25 Synthesis of desialylated and deglycosylated EPO and Fc-EPO protein by enzyme treatment

Sialic acid residues were removed from EPO and Fc-EPO by treatment with neuraminidase. Fc-erythropoietin protein at 500 micrograms/ml was treated with 0.1 units/ml of enzyme (Roche Biologicals) in a buffer containing 50 mM sodium  
30 acetate, 4 mM calcium chloride, 100 micrograms/ml bovine serum albumin at pH 5.5 for various times at 37°C.

Data in Figure 2 illustrate that human IgG2 Fc-EPO treated with neuraminidase has an increased activity. For example, Fc-EPO that has been treated with

neuraminidase for 22 hours has an activity equal to that of about 2 to 5 times as much normally sialylated Fc-EPO control protein.

To completely remove the N-linked sugar moieties, N-glycosidase treatment was used. Fc-erythropoietin protein at 500 micrograms/ml was treated with 0.02

- 5 units/ml of enzyme (Roche Biologicals) in a buffer containing 50 mM phosphate pH 7.8 for various times at 37°C. Alternatively, a buffer containing 50 mM phosphate pH 7.8, 20 mM EDTA, 1% Triton X-100, 1% beta-mercaptoethanol, and 0.1% SDS is used.

10 **Example 4.**

Characterization of Fc-EPO and deglycosylated Fc-EPO

To characterize the deglycosylated forms of Fc-EPO that were generated by enzyme treatment or expression in mutant cell lines, SDS-PAGE and isoelectric focusing experiments were carried out. As determined by SDS-PAGE, the Fc-  
15 EPO protein that was deglycosylated by N-glycosidase treatment showed significantly faster mobility (Figure 1).

The Fc-EPO protein is a dimer with four N-glycosylation sites and one O-glycosylation site in each subunit, for a total of ten glycosylation sites and 36 sialic acid residues. Each one of these sites is incompletely modified, so that Fc-  
20 EPO has many forms when analysed by IEF.

When Fc-EPO is treated with neuraminidase, certain IEF bands disappear and others appear, consistent with the removal of sialic acid by this enzyme.

Similarly, Fc-EPO that is produced by from CHO-lec2 cells has a smaller number of less acidic forms.

25

**Example 5.**

ELISA procedures

ELISAs were used to determine the concentrations of protein products in the supernatants of MTX-resistant clones and other test samples. The amounts of  
30 human Fc- and murine Fc-containing proteins were determined by the anti-huFc ELISA and the anti-muFc ELISA, respectively.

ELISA plates were coated with AffiniPure Goat anti-Human IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA) at 5 µg/mL in PBS, and 100 µl/well in 96-well plates (Nunc-Immuno plate Maxisorp). Coated plates were

covered and incubated at 4°C overnight. Plates were then washed 4 times with 0.05% Tween (Tween 20) in PBS, and blocked with 1% BSA/1% goat serum in PBS, 200 µl/well. After incubation with the blocking buffer at 37°C for 2 hrs, the plates were washed 4 times with 0.05% Tween in PBS and tapped dry on paper  
5 towels.

Coated plates were incubated with test samples diluted to the proper concentrations. Sample buffer contains 1% BSA, 1% goat serum and 0.05% Tween in PBS. A standard curve was prepared with a chimeric antibody (with a human Fc), the concentration of which was known. To prepare a standard curve,  
10 serial dilutions are made in the sample buffer to give a standard curve ranging from 125 ng/mL to 3.9 ng/mL. The diluted samples and standards were added to the plate, 100 µl /well and the plate was incubated at 37°C for 2 hr. After incubation, the plate was washed 8 times with 0.05% Tween in PBS. To each well was then added 100 µl of the secondary antibody, the horseradish  
15 peroxidase-conjugated anti-human IgG (Jackson Immuno Research), diluted according to manufacturers instruction in the sample buffer. After incubation at 37°C for 2 hr, the plate was washed 8 times with 0.05% Tween in PBS. Substrate solution have been added to the plate at 100 µl/well. The substrate solution was prepared by dissolving 30 mg of OPD (o-phenylenediamine  
20 dihydrochloride, 1 tablet) into 15 mL of 0.025 M Citric acid/0.05 M Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 5, which contained 0.03% of freshly added H<sub>2</sub>O<sub>2</sub>. The color was allowed to develop for 30 min. at room temperature in the dark. The developing time is subject to change, depending on lot to lot variability of the coated plates, the secondary antibody, etc. Watch the color development in the standard curve  
25 to determine when to stop the reaction. The reaction was stopped by adding 4N H<sub>2</sub>SO<sub>4</sub>, 100 µl/well. The plate was read by a plate reader, which was set at both 490 and 650 nm and programmed to subtract the background OD at 650 nm from the OD at 490 nm.

The procedure for the anti-muFc ELISA is similar, except that ELISA plate was  
30 coated with AffiniPure Goat anti-murine IgG (H+L) (Jackson Immuno Research) at 5 µg/mL in PBS, and 100 µl/well; and the secondary antibody was horseradish peroxidase-conjugated goat anti-mulgG (Southern Biotechnology Assoc., Birmingham, AL).

**Example 6:**In vitro activity of human Fc-EPO fusion proteins

The methods below were and are used to test the activity of human Fc-EPO proteins produced by transient and stable expression. The amount of each fusion protein in the cell culture supernatant was first determined by ELISA and used to set up a dose-response curve. The activities closely corresponded to what was found with the Fc-EPO fusion proteins and described above.

Specifically, the EPO activity of a human Fc-EPO and asialo- human Fc-EPO molecules were tested in the TF-1 cell proliferation assay, following a standard procedure known to those practiced in the art of molecular immunology (Hammerling et al. [1996] J. Pharmaceutical and Biomedical Analysis 14:1455-1469; Kitamura et al. [1989] J. Cellular Physiol. 140:323-334). The human TF-1 cell line proliferates in response to EPO or other cytokines and growth factors. TF-1 cells in active log-phase growth were washed twice in medium lacking EPO and plated at about  $1 \times 10^4$  cells per well in microtiter wells in the presence of various amounts of commercial EPO or Fc-EPO fusion protein with or without sialic acids. Cells were incubated in the presence of various test proteins for 48 hours, and 0.3 microCuries of  $^3\text{H}$ -thymidine was added ten hours before determining levels of radioactive incorporation. The various EPO and Fc-EPO fusion proteins stimulated incorporation of  $^3\text{H}$ -thymidine into cells in a dose-dependent manner, and were about equally effective in stimulating incorporation of  $^3\text{H}$ -thymidine on a per mole basis.

These results indicated that the *in vitro* biological activity of Fc-EPO increased upon desialylation by neuraminidase. The results also indicate that Fc-EPO fusion proteins, with or without sialylation, had activity similar to human EPO. Specifically, Figure 1 indicates that the biological activity of normally sialylated human IgG2 Fc-EPO was about 2 to 5-fold less than that of enzymatically desialylated human IgG2 Fc-EPO, and the activities of these Fc-EPO fusion proteins were similar to the NIBSC EPO on a per mole basis.

**Example 7**Site-directed mutagenesis of unglycosylated EPO

Mutations that increase the activity of unglycosylated EPO are introduced into the Fc-unglycosylated EPO fusion protein as follows. A DNA sequence encoding unglycosylated EPO is constructed as described in Example 1, except that one

AATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTACTCCGCAATTCCTCC

The following mutations are introduced by this procedure: Gly<sub>101</sub>Ala, Arg<sub>143</sub>Ala, Ser<sub>146</sub>Ala, and Asn<sub>147</sub>Ala. These mutations most likely have the effect of increasing the activity of Fc-EPO by increasing its affinity for the EPO receptor.

In other versions, cysteine residues are inserted and removed by substitution as described in Example 13. The resulting protein is more stable and more efficiently expressed, especially when combined with the mutations described above.

## 20 Testing site-directed mutants for activity

### Example 9:

To generate mutant forms of Fc-unglycosylated EPO without pre-selection, one of the following procedures is used. For example, the mature unglycosylated EPO coding sequence is synthesized as described in Example 1, except that 10 separate pools are generated. In the first pool, Oligo 1 and its reverse

complement are synthesized with mixtures of oligonucleotide precursors so that each nucleotide has a 3% chance of being mutant. As a result, on average, each oligonucleotide will have 1 to 2 amino acid substitutions. Similarly, in the second pool, Oligo 2 and its reverse complement are synthesized with mixtures of  
5 oligonucleotide precursors so that each nucleotide has a 3% chance of being mutant, and so on.

After ligation and transformation into *E. coli*, about 20 colonies are picked for each pool. DNA is made from each of the 20 transformants, and then separately introduced into a mammalian cell line such as BHK cells. Supernatant from each  
10 set of transiently transfected cells is then tested for EPO activity as described in Example 9.

Particular transfected BHK lines are found to produce EPO with greater specific activity. The corresponding DNA sequences of the mutant coding regions are determined. Based on these mutations and mutations identified in the Examples  
15 above, multiply mutant coding sequences are constructed. The corresponding multiply mutant proteins are expressed, and certain forms are found to have specific activities that are even greater than the individually mutant "parental" forms.

## 20 **Example 10**

### Pharmacokinetic data

At present, erythropoietin is normally administered to patients three times per week (Physicians' Desk Reference [1996] "EPOgen: EPOetin Alfa", p. 489-496). The serum half-life of intravenously administered erythropoietin is about 4 to 13  
25 hours. After subcutaneous administration of erythropoietin, serum levels peak within 5 to 24 hours. It would be advantageous to have a protein that stimulates red blood cell production but with a longer serum half-life than erythropoietin, so that dosing could be less frequent.

## **Example 11**

### 30 Pharmacokinetics of Fc-EPO fusion proteins

The human EPO protein and certain human Fc-EPO fusion proteins were tested for their pharmacokinetic behavior following intravenous injection into Balb/c mice. Blood was collected from mice by retro-orbital bleeding and stored at 4°C in Eppendorf micro-centrifuge tubes. ELISA methods were used to measure the

amount of human antibody-related proteins, such as the human Fc region, remaining in the blood at various time points. The ELISA measuring human antibody used an antibody against human H and L chains for capture and an anti-human Fc antibody for detection. A Western blot was used to verify that the Fc-erythropoietin fusion protein retained the correct size and was not degraded. As an alternative method to detect intact Fc-erythropoietin fusion protein moieties, a modified ELISA method was used. This fusion protein-specific assay uses the same first capture step, but an anti-human EPO antibody for detection. To detect EPO alone, both the capture antibody and the detection antibody are specific for human EPO. For example, the human EPO detection kit is used. The Fc-EPO fusion had a serum half-life of about 2 to 4 hours in these experiments. In contrast, the serum half-life of certain more elaborately engineered Fc-EPO fusions is tested and found to be much longer. For example, an intact antibody-EPO fusion is tested and has a serum half-life in mice of about 10 to 20 hours or longer. The serum half-life of intact asialo-EPO was determined to be very short. As determined by Western blotting using antibodies against human IgG as a probe, the erythropoietin moiety of the asialo-Fc-EPO fusion protein is rapidly degraded while the Fc moiety is relatively stable and retained in the serum. These results indicate that only certain Fc-EPO fusion proteins have long serum half-lives, and that the Fc moiety is not universally sufficient to extend the serum half-life of a protein.

### Example 12

#### In vivo activity of Fc-EPO fusion proteins

The *in vivo* activity of the human Fc-EPO fusion protein was tested and compared with that of intact human EPO. On a per mole basis, the activity of Fc-erythropoietin is similar to that of intact human EPO using an assay that measures stimulation of red blood cell production within a short period after administration.

The activity of human EPO and IgG2 Fc-EPO was assayed in the normocythaemic mouse assay. One week before the beginning of the assay procedure, 8-week old male mice of the strain B6D2F1 were distributed into cages with six animals per cage. Within each cage group, each animal was



injected with 0.5 mls of 10, 20 or 40 micrograms/ml of either erythropoietin or Fc-EPO, where the dose of Fc-EPO was measured by calculation of the amount of EPO monomers as determined by ELISA. For each experiment, 8 animals were used for each dose group.

- 5 Four days after the injections, blood samples were collected and the number of reticulocytes per 30,000 red blood cells was determined as follows. One microliter of whole blood was added to 1 milliliter of 0.15 micromolar acridine orange. After staining for 3 to 10 minutes, the reticulocyte count was determined microfluorometrically in a flow cytometer, by analysis of the red fluorescence
- 10 histogram. The following data was obtained:

Table: 1

Number of reticulocytes per 30,000 red blood cells

Dose	EPO			IgG2 Fc-EPO		
	10	20	40	10	20	40
Average	1177	1422	1820	1036	1322	1732
Standard deviation	119	102	197	75	83	178

- 15 In a variation of this assay, mice are dosed with erythropoietin, Fc-Erythropoietin, Ig-Erythropoietin, and various other forms of Fc-EPO containing mutations, truncations, or altered glycosylation patterns. Reticulocytes are measured as described above, except that blood is sampled at 4 days, 5 days, 6 days, and 7 days following the injection of the test protein. Performing the experiment in this
- 20 manner gives an indication of the functional pharmacokinetics of the test protein. It is found that certain forms of Fc-EPO, such as intact Ig-EPO, show functional activity over a longer period of time than normal EPO.

- As an alternative method for measuring EPO activity, the *in vivo* activity of human EPO and Fc-EPO proteins is tested by the starved rat assay (Goldwasser E. and
- 25 Gross M. Erythropoietin: assay and study of its mode of action. Methods Enzymol. [1975] 37 Pt B:109-21). Male Sprague-Dawley rats weighing about 215 to 250 grams (about 9 weeks old) are deprived of food on day 1. They are

then injected i.v. with 2 mls of test material on days 2 and 3. The rats are divided into groups of five rats each. To generate a standard curve, one group is injected with physiological saline, and four other groups are injected with 1.0, 1.5, 2.0, or 3.0 Units of erythropoietin per rat, where 1.246 Units corresponds to 1 nanogram of glycoprotein (=26.7 femtomoles; check this). On day four, 28 hours after the second injection, the rats are injected i.p. with 1.0 microCuries of  $^{59}\text{Fe}^{3+}$ , in physiological saline buffered with citrate. Sixteen to eighteen hours after the  $^{59}\text{Fe}^{3+}$  injection, the rats are anaesthetized and bled by cardiac puncture, using heparinized syringes. One ml of blood is withdrawn for counting the radiolabel, and a microhematocrit tube is also filled with blood. The animal is weighed. The per cent of the injected  $^{59}\text{Fe}^{3+}$  incorporated into the total red cell mass is calculated, assuming that the blood constitutes 5% of the weight of the animal. The hematocrits are recorded, and data from rats with hematocrits of less than 50 are discarded. The data are evaluated by subtracting the mean of the saline control group from the mean of each test group to obtain the per cent incorporation that is stimulated by EPO or Fc-EPO fusion proteins.

As another alternative, the *in vivo* activity of human EPO and Fc-EPO proteins is tested by the plethoric mouse assay (Goldwasser E. and Gross M. *ibid*). In this assay, mice are given a surplus of red blood cells so that erythropoiesis is suppressed. Methods of creating the plethora of red blood cells include exposure to low pressure (about 0.5 atmospheres), exposure to low oxygen at normal pressure, exposure to a low level of carbon monoxide, or exposure to a gradually decreasing  $\text{O}_2$  partial pressure. The mice may be injected s.c. with 2.5 mg of iron-dextran before exposing them to the hypoxic stress, in order to insure that the mice have enough iron for the increased hemoglobin synthesis. Mice are returned to a normal oxygen environment on day 1 and injected with  $^{59}\text{Fe}^{3+}$  on day 8.

Alternatively, mouse red cells are injected into the mice. For example, 1 ml of packed, washed isologous red blood cells is injected i.p. on days 1 and 3. Test samples include a saline control and standard doses of 0.05, 0.10 and 0.20 Units per mouse. These are injected on days 5 and 6, and  $^{59}\text{Fe}^{3+}$  is injected on day 7, and the mice are bled on day 10. One ml of blood is counted. Some blood is used for a hematocrit. The mice are weighed. The per cent of body weight

represented by blood is assumed to be 8%. If a hematocrit is below 55, data for that mouse are not used.

Variations on these procedures, as well as other procedures, can also be used to determine the *in vivo* activity of various forms of the EPO protein.

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### Example 13.

#### Construction and expression of Fc-EPO variants containing altered patterns of disulfide bonding.

Mutations that alter the disulfide bonding pattern of the EPO moiety within Fc-EPO were introduced as follows. The alterations His<sub>32</sub>Gly, Cys<sub>33</sub>Pro, Trp<sub>88</sub>Cys, and Pro<sub>90</sub>Ala were introduced into human Fc-EPO by standard site-directed mutagenesis techniques. This protein was termed Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>). Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) was expressed in mammalian cells by procedures analogous to those described in the preceding examples. Fc-EPO and Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) protein were purified using a Staph A protein column as described in Example 2.

Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) was found to be 1.5- to 2-fold more active than Fc-EPO in cell-based assays that measured proliferation of TF-1 cells. To investigate why Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) was more active than Fc-EPO, each purified protein was examined by HPLC. Figures 4 and 5 show typical results. About 1/3 to 1/2 of the Fc-EPO protein migrated through the column with an apparent molecular weight of about 100,000 Daltons, which is the predicted molecular weight of dimeric Fc-EPO, but the remaining 1/2 to 2/3 of the Fc-EPO protein migrated with a much higher molecular weight, indicating that the Fc-EPO was in an aggregated state (for example, as in Figure 4). SDS-PAGE, performed under denaturing and reducing conditions, indicated that this high-molecular weight material was not due to contamination with other proteins. In contrast, about 95% of the Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) protein migrated through the HPLC column with an apparent molecular weight of 100,000 Daltons, and only about 5% of the Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) was in an apparently aggregated state (for example, Figure 5). Standard HPLC conditions were used.

To further investigate the enhanced stability of Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>), both Fc-EPO and Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) were treated with N-glycanase, which removes the three N-linked oligosaccharides from erythropoietin. Standard digestion conditions were used in accordance with the manufacturer's instructions. Under these conditions, the N-linked oligosaccharides were completely removed from Fc-EPO and Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) within 1 hour, as determined by SDS-PAGE – incubation longer than 1 hour had no effect on the migration of the Fc-EPO proteins, but it was found that further incubation under conditions of digestion with N-glycanase did cause Fc-EPO. However, not Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>), to rapidly lose biological activity, as described below.

After incubation of Fc-EPO or Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) in the presence of N-glycanase for various times, the reaction was terminated by freezing at –20°C and TF-1 cells were incubated with various dilutions of treated Fc-EPOs. Stimulation of <sup>3</sup>H-thymidine incorporation was measured and compared with the NIBSC Erythropoietin standard. Results were obtained as shown in the table below.

**Table II.** *Effect on biological activity of digestion of Fc-EPO and Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) with N-glycanase.*

Treatment	Specific Activity	
	(International units per mg of EPO moiety within a fusion protein)	
	Fc-EPO	Fc-EPO (Cys <sub>29</sub> -Cys <sub>88</sub> )
None	55,000	82,000
N-glycanase treatment (0 min)	55,000	89,000
N-glycanase treatment (15 min)	34,000	82,000
N-glycanase treatment (60 min)	3,290	67,000
N-glycanase treatment (120 min)	1,066	67,000

These results indicated that Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) was much more stable upon N-glycanase treatment than Fc-EPO. Without wishing to be bound by theory, it may be that the N-glycanase buffer conditions, namely phosphate-buffered saline, are destabilizing to the deglycosylated EPO moiety within Fc-EPO, or that the N-glycanase is contaminated with proteases that inactivate the EPO moiety.

Introduction of the mutations in Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) allows the formation of a disulfide bond between Cys<sub>29</sub> and Cys<sub>88</sub> of EPO. Similarly, introduction of analogous mutations into intact, unfused human EPO causes the formation of a disulfide bond between Cys<sub>29</sub> and Cys<sub>88</sub>. The disulfide bond is identified by

5 cleavage with a site-specific endoprotease such as trypsin under non-reducing conditions, followed by analysis of resulting peptides using mass spectrometry or HPLC analysis.

For example, the following set of experiments with controls is performed. Human

10 Fc-EPO, human Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>), human EPO, and human EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) are cleaved with trypsin in both reducing and non-reducing conditions. These eight samples are analyzed by mass spectrometry. Trypsinized non-reduced human Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) and human EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) each give a peak with a high molecular weight, corresponding to

15 EAENITTGCAEGPSLNENITVPDTK + GQALLVNSSQPCEPLQLHVDK with two N-linked glycosylations. Because of its large size and heterogeneity due to the presence of two N-glycosylations, this peak is easily distinguished from the other peaks. This peak is not found in reduced samples or in samples derived from non-mutant human EPO or non-mutant human Fc-EPO. As a further diagnostic test, samples

20 are incubated with N-glycanase before treatment with trypsin. In the samples treated with N-glycanase, the peak corresponding to EAENITTGCAEGPSLNENITVPDTK (pos. 21 – 45, Seq. Id. No. 2) + GQALLVNSSQPCEPLQLHVDK (pos. 77 – 97, Seq. Id. No. 2) is shifted to the size predicted by the molecular weights of the amino acids alone.

25

The Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) is tested further and found to be advantageous in other ways. For example, Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) has superior pharmacokinetic properties when tested in mice, humans, or other mammals. Lyophilized forms of Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) and EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) are more stable than the

30 corresponding Fc-EPO and EPO proteins. In long-term stability studies, such as studies of remaining biological activity after extended incubation at elevated temperatures, Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) and EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) are more stable than the corresponding Fc-EPO and EPO proteins. Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) and

EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) are more resistant to proteases than the corresponding Fc-EPO and EPO proteins.

In addition, it is sometimes useful to introduce mutations into the EPO moiety that are advantageous in certain aspects but that also decrease the stability of the EPO moiety. In such cases, it is useful to also introduce one or more mutations that cause the formation of a disulfide bond between Cys<sub>29</sub> and Cys<sub>88</sub>. The effect of the additional disulfide bond is to enhance the stability of the mutated EPO. For example, mutation of Gly<sub>101</sub> → Ala, Arg<sub>143</sub> → Ala, Ser<sub>146</sub> → Ala, and Asn<sub>147</sub> → Ala increases the signaling activity of EPO. Mutations of this type have advantages with regard to certain properties of EPO, but destabilize the protein for purposes of pharmaceutical development.

The advantageous properties of a mutation or mutations that cause the formation of a disulfide bond between Cys<sub>29</sub> and Cys<sub>88</sub> in EPO are also observed in intact EPO without an attached Fc moiety, and also in other forms of EPO such as fusion proteins of EPO to other moieties, forms of EPO that have reduced, increased, or qualitatively altered glycosylation levels, and so on.

In a similar set of experiments, an expression plasmid encoding human Fc-EPO protein containing the mutations Arg<sub>139</sub> → Cys and Cys<sub>29</sub> to another amino acid such as Ala, Val, Leu, or Ile is constructed analogously to the construction of Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>). Analysis by protease treatment and mass-spectrometry indicates that this protein contains a disulfide bond between Cys<sub>33</sub> and Cys<sub>139</sub> and is therefore termed Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>). An analogous expression plasmid encoding human EPO containing the mutations Arg<sub>139</sub> → Cys and Cys<sub>29</sub> to another amino acid such as Ala, Val, Leu, or Ile is also constructed.

Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) has a number of advantageous properties. For example, Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) is primarily in the normal, dimeric form and is less aggregated than human Fc-EPO. For example, when purified Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) is analyzed by HPLC, most of the material migrates with an apparent molecular weight of about 100 kD. Another advantageous property is that Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) is more active than human Fc-EPO. Without wishing to be

bound by theory, it is most likely that the 100 kD form of both Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) and human Fc-EPO is the active form, and the forms with a high apparent molecular weight, as determined by HPLC, have little or no activity. Even though the Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) and Fc-EPO (Cys<sub>29</sub>-Cys<sub>88</sub>) have increases in activity  
5 of 25% to 100%, this improved activity is economically significant because these proteins are expensive to make and large quantities are used to treat the large population of patients who are anemic.

Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) also shows improved pharmacokinetics compared to  
10 human Fc-EPO. Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) also shows improved long-term stability in solution and in a lyophilized form as compared to human Fc-EPO.

Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) also has the advantageous property that, in the presence of additional alterations or mutations that destabilize human Fc-EPO, the protein  
15 has significantly enhanced stability.

The advantageous properties of Fc-EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) are also observed with EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) without the Fc moiety. For example, EPO (Cys<sub>33</sub>-Cys<sub>139</sub>) has enhanced stability, improved activity, superior pharmacokinetics, improved long-  
20 term stability, and significantly enhanced stability in the presence of additional destabilizing alterations.

Other useful forms of human Fc-EPO and human EPO include multiply mutant proteins that have disulfide bonds between Cys<sub>29</sub> and Cys<sub>88</sub> as well as between  
25 Cys<sub>33</sub> and Cys<sub>139</sub>. For example, EPO (Cys<sub>29</sub>-Cys<sub>88</sub>+Cys<sub>33</sub>-Cys<sub>139</sub>) has enhanced stability, improved activity, superior pharmacokinetics, improved long-term stability, and significantly enhanced stability in the presence of additional destabilizing mutations.

Human Fc-EPO and human EPO are engineered to have advantageous  
30 properties by the introduction of other disulfide bonds. The design of such disulfide bonds can be guided by the known structure of human EPO, which has been determined using X-ray crystallography and NMR. For example, Ala<sub>22</sub> and Phe<sub>142</sub> of human EPO or human Fc-EPO are each replaced by cysteine, and a disulfide bond forms between these new cysteines. To compensate for the empty

For example, Val<sub>74</sub> is replaced with Phe, Leu, Tyr, Ile, or Met. The resulting protein with an additional disulfide has enhanced stability, improved activity, superior pharmacokinetics, improved long-term stability, and significantly enhanced stability in the presence of additional destabilizing mutations.

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